

Glycopeptide and glycan analysis of monoclonal antibodies using a microfluidic-based HPLC-Chip coupled to an Agilent Accurate-Mass Q-TOF LC/MS

Application Note

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Abstract

In this study, we have analyzed the glycopeptide and glycan moiety of a monoclonal antibody (mAb) using an Agilent HPLC-Chip coupled with an Agilent Accurate-Mass Q-TOF LC/MS. The tryptic peptide map of the mAb was generated and the glycopeptide was assigned using accurate mass measurement. The enzymatically released glycan moiety from the mAb was extracted and analyzed on an HPLC-Chip coupled with a Q-TOF. Diagnostic oxonium ions from a glycan moiety were further used to identify the glycopeptide and the glycan. The superior mass accuracy of the LC/MS platform, combined with the powerful data processing capabilities of Agilent MassHunter and BioConfirm software, enabled identification of the glycopeptides and glycan moiety attached to the protein complex.



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Introduction

Glycosylation of monoclonal antibodies is one of the common post translation modifications. The glycan moieties have a key role in immunogenicity, effector function efficacy, and clearance of the mAbs. The glycosylation of antibodies is influenced by the cell in which it is produced as well as cell culture conditions including pH, temperature, medium, etc. In this application note, purified mAb was subjected to proteolytic digestion followed by peptide separation and accurate mass determination on an HPLC-Chip coupled to an Accurate-Mass Q-TOF LC/MS. BioConfirm software was used to map the glycation site on the mAb. The accurate mass measurement in MS only mode of the Q-TOF allowed for easy assignment of the glycopeptide. Furthermore, from MS/MS data, characteristic diagnostic sugar oxonium ion fragments were used to confirm the glycopeptide. Glycan analysis was also executed by enzymatically releasing the glycan moiety in solution followed by separation on a graphitized carbon column and accurate mass determination on the HPLC-Chip coupled to a Q-TOF. This application note continues our studies toward complete characterization of monoclonal antibodies using advanced Agilent platforms ideal for the biopharma market [1, 2].

Materials

Immunoglobulin G (IgG) was obtained from ProMab Biotechnologies, Inc (USA). DL-Dithiothreitol (DTT), iodoacetamide, and Tris (hydroxymethyl)-aminomethane (Tris Base) were purchased from Sigma Aldrich. High quality sequence grade trypsin and PNGase F were obtained from Agilent Technologies, Inc. Acetone was purchased from Ranbaxy Laboratories Limited (India) and methanol was purchased from Lab-Scan Analytical Sciences (India). PNGase F was prepared internally for Agilent use and was not commercially available.

Sample pre-treatment:

Reduction and alkylation of an antibody under denaturation conditions

Before the digestion of the mAb with trypsin, the disulfides were reduced and alkylated under denaturation conditions. This pretreatment ensured that the monoclonal antibody was completely denatured and soluble, allowing the protease to access its substrate efficiently [3, 4].

The mAb was lyophilized, reconstituted in 2 μ L of 8 M urea in 0.25 M Tris buffer (pH 7.6) containing DTT, and then incubated at 37°C for 30 min. To this solution, 2 μ L of a solution containing iodoacetamide in 8 M urea in 0.25 M Tris buffer (pH 7.6) was added, and the sample was incubated at ambient temperature in the dark for 15 min. The solution was diluted with 160 μ L of 0.25 M Tris buffer (pH 7.6) before digestion with trypsin.

Trypsin digestion

Trypsin was added to the above pretreated mAb solution at a ratio of 20:1 (protein to protease w/w). The reaction was kept for overnight incubation at 37°C. The enzymatic activity was quenched by adding 1 μ L of 10% formic acid solution. The samples were either immediately analyzed by LC/MS/MS or stored at -80°C until LC/MS/MS analysis.

Glycan release and extraction

The mAb was deglycosylated by treatment with PNGase F in 0.25 M Tris buffer (pH 7.6) overnight at 37°C. The deglycosylated protein including the freed glycan was precipitated using cold acetone (80%) for 30 min at -20°C. The acetone precipitated protein was centrifuged as a pellet and the supernatant was discarded. Next, a 60% methanol/water mixture was added to selectively extract the free glycan from the acetone precipitate. The methanol solution was centrifuged to extract the glycan in the supernatant, leaving the deglycosylated protein as a pellet. This methanol extract was vacuum-dried and used further for LC/MS analysis.

Instrumentation

An Agilent 1200 Series HPLC-Chip/MS Interface (PN: G4240A) was coupled with an Agilent 6520 Accurate-Mass Q-TOF LC/MS System for LC/MS analyses.

LC Parameters:

HPLC-Chip: Two different kinds of chips were used in this study:

A) 5 μ m, ZORBAX 300SB-C18 (300Å), 40 nL enrichment column, and a 75 mm x 43 mm analytical column (PN: G4240-62001).

B) 40 nL enrichment column, 43 mm x 75 μ m analytical column packed with Graphitized Carbon (PN: G4240-62003).

Flow rate: 3 μ L/min from an Agilent 1200 Series Capillary Pump (PN: G1382A) to the enrichment column and 600 nL/min from an Agilent 1200 Series Nanoflow LC pump (PN: G2226A) to the analytical column.

Solvents: 0.1% formic acid in water (A); 90% acetonitrile in water with 0.1% formic acid (B).

Sample Loading: Agilent 1200 Series Capillary Pump at 3% B.

Amount of sample injected onto the chip: 50 ng of the protein digest.

Sample analysis: Gradient with an Agilent 1200 Series Nanoflow LC pump as shown below.

Time (min)	B (%)
Initial	3
30	50
32	95
34	95
34.10	3

Stop time: 36 min

MS Parameters:

Spectra were recorded in positive ion and in centroid mode.

Vcap: 1900 V and drying gas flow of 5 L/min at 325°C was used.

Fragmentor voltage: 175 V

Data were acquired at high resolution 4 GHz: for MS only mode, the range was 300–3,200 m/z ; for MS/MS, spectra were acquired in auto MS/MS mode with the following parameters: MS scan, m/z 300–3,200 at 1 spectra/sec and MS/MS scan, m/z 50–3,000 at 3 spectra/sec. Precursor selection criteria: maximum of 3 precursors per cycle above threshold 1,000, active exclusion after 2 spectra for 0.5 min, and preferred charge state selections in the following order: 2, 3, >3, unknown. Collision Energy (CE) was set at 3.7 V/100 Da with 2.5 V as offset. An internal mass calibration sample was infused continuously during the LC/MS runs. This internal reference mass system allowed accurate, and automated mass calibration correction during the LC/MS runs.

Data analysis: The data obtained from LC/MS and MS/MS were analyzed using features contained in the following software packages: Agilent MassHunter Qualitative Analysis, Agilent MassHunter BioConfirm, and Agilent Spectrum Mill MS Proteomics Workbench.

Molecular feature extraction: The raw data (chromatograms) were processed using the Molecular Feature Extractor (MFE) algorithm within Agilent MassHunter Qualitative Analysis software.

Define and match sequence: Both the light and heavy chain sequences were digested *in silico* using trypsin with 2 missed cleavages containing preferred glycosylation modifications to generate a theoretical peptide digest list. The compounds extracted using MFE were matched against this list.

Results and discussion

Glycopeptide analysis

The purified mAb was subjected to trypsin digestion and a peptide map was generated (2). **Figure 1** shows excellent chromatographic resolution for a base peak chromatogram (BPC) of peptide mapping using a nanospray-based HPLC-Chip. The glycopeptides eluted at 4.42 min (**Figure 1A**). Using MFE and the BioConfirm sequence editor, peptide masses from the LC/MS run were matched with the theoretical digest at a 5 ppm error with preferred glycosylation modifications included in the sequence of an antibody. **Figure 2** shows a snapshot of the BioConfirm window for the trypsin digest of a mAb and the matched glycopeptide. The glycopeptide matches to within 2.14 ppm. The mass spectrum of the glycopeptide is shown in **Figure 1B** with sequence. A minor amount of G1F is also detected as shown in Figure 2.

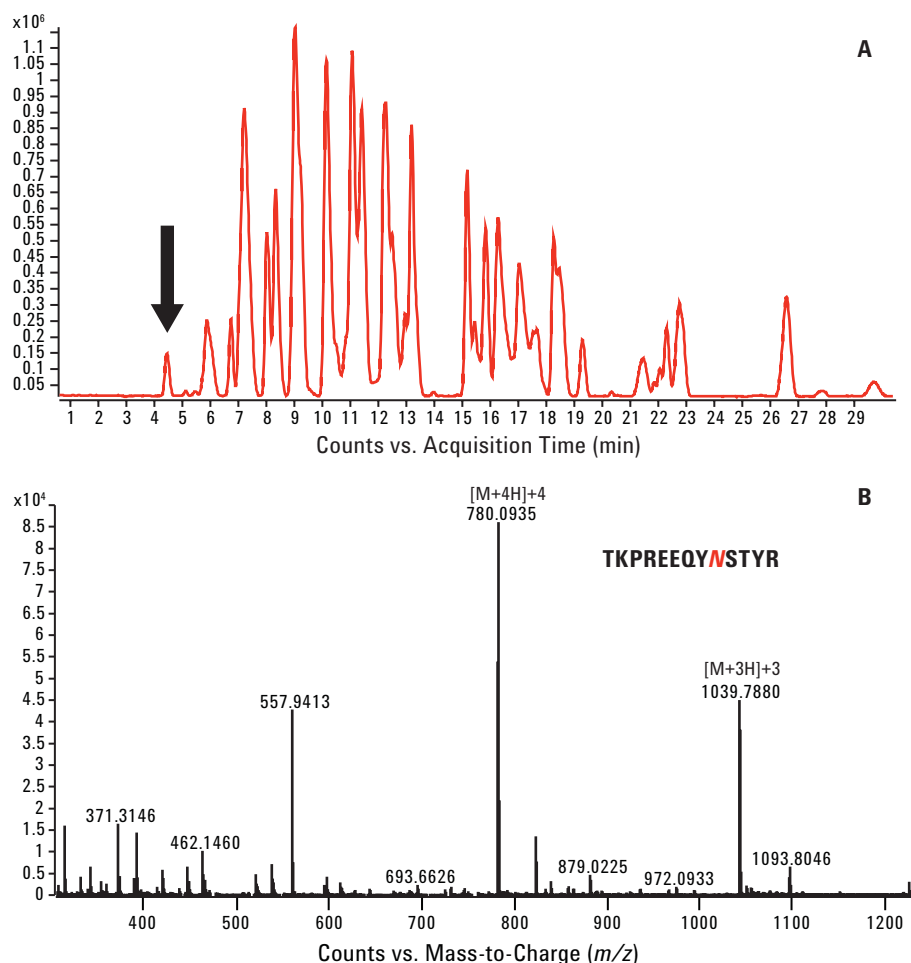


Figure 1: (A) Base peak chromatogram (BPC) of a trypsin-digested mAb on a C_{18} HPLC-Chip. The arrow marked on the peptide map shows elution of the glycopeptide. (B) Mass spectrum of the glycopeptides with sequence.

File	RT	Mass	Algorithm	Ions	Height	Sequence	Tgt Seq Mass	Match Diff (ppm)	Mode
promabthyme.d	4.401	3277.3928	Find by Molecular...	29	29748	TKPREEQYNSTYR	3277.388	1.48	G1.
promabthyme.d	4.452	2795.0925	Find by Molecular...	20	2293	EEQYNSTYR	2795.0914	0.37	G1.
promabthyme.d	8.321	2796.0818	Find by Molecular...	14	744	EEQYNSTYR	2796.0755	2.27	G1.
promabthyme.d	4.42	3115.3418	Find by Molecular...	71	171293	TKPREEQYNSTYR	3115.3351	2.14	G0.
promabthyme.d	4.472	2633.0399	Find by Molecular...	50	17478	EEQYNSTYR	2633.0386	0.48	G0.
promabthyme.d	18.303	4842.2266	Find by Molecular...	14	1029	EEQYNSTYRVSVL	4842.2262	-0.32	G0.
promabthyme.d	8.304	1317.5942	Find by Molecular...	12	746060	AEDTAVVYCVK	1317.5911	2.38	Alk.
promabthyme.d	13.046	2170.0786	Find by Molecular...	4	1210	AEDTAVVYCVKDLGI	2170.0841	-2.53	Alk.
promabthyme.d	13.797	2013.9842	Find by Molecular...	9	1176	AEDTAVVYCVKDLGI	2013.963	0.59	Alk.
promabthyme.d	15.554	3068.4541	Find by Molecular...	15	6314	DNSKNTLYQMSL	3068.4583	1.88	Alk.
promabthyme.d	15.755	2624.2706	Find by Molecular...	6	4440	NTLYQMSLRAED	2624.2615	3.47	Alk.
promabthyme.d	18.857	2220.6534	Find by Molecular...	81	814	NTLYQMSLRAED	2220.6534	0.00	Alk.

Figure 2: A BioConfirm window in MassHunter Qualitative Analysis software showing the compound list of matched tryptic digest with theoretically generated peptides of a mAb. The matched major glycopeptide is highlighted in the compound list. The arrow points to the minor form of glycopeptide (G1F) observed in the tryptic digest of mAb.

Analysis of characteristic diagnostic sugar oxonium ion fragments (B-type oxonium ions), resulting from the MS/MS fragmentation of the N-linked glycan on the glycopeptide, was used to identify and quickly confirm the presence of glycopeptides in the LC/MS runs. In this study, the presence of glycopeptide was confirmed using the extracted ion chromatogram (EIC) at m/z 204.085 (within an error limit of 2 ppm), corresponding to one of the intense diagnostic sugar oxonium fragment ions as shown in Figure 3. From Figure 3, it can be determined

that a peak was observed at 4.42 min, and that this “glycopeptide” peak agrees with the assignment of the correct mass (Figure 1A).

The product ion spectrum of the triply charged precursor of the glycopeptide showed major ion signals in the lower and mid m/z range for glycan fragmentation and some minor ones for glycopeptides (data not shown). The peptide sequence (TKPREEQY**N**STYR) has only one asparagine residue / **NXT** motif, which is a known site of glycosylation in mAbs. Therefore, it was

deduced that the glycan is attached to the asparagine residue of the **NST** motif in the peptide sequence. To further characterize the glycan moiety of the mAb, the glycan was enzymatically released from the mAb.

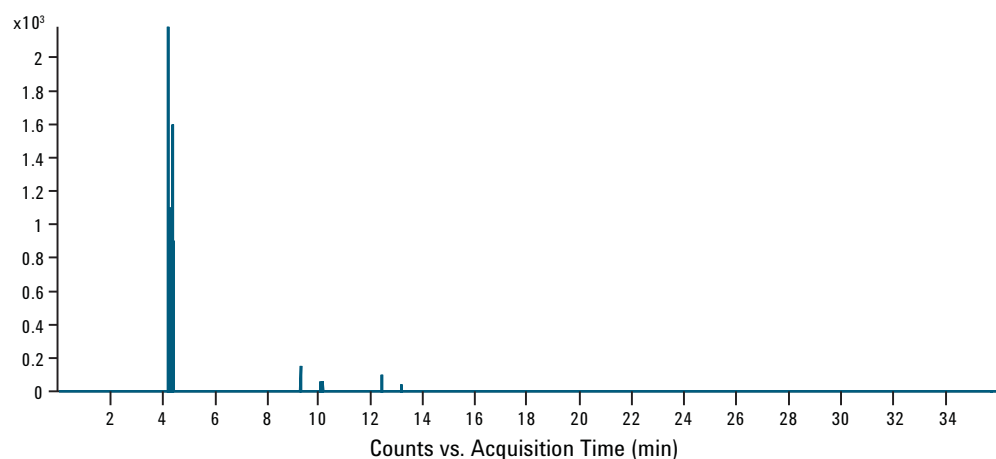


Figure 3: Extracted ion chromatogram MS (all) at m/z 204.084 for the trypsin-digested mAb using a C_{18} HPLC-Chip.

Glycan analysis

The enzymatically released glycan moiety from the mAb was extracted using a water/organic solvent extraction procedure. The released glycan was analyzed on graphitized carbon material contained within an HPLC-Chip. Graphitized carbon chips retain

the glycan better than the regular C18 reverse phase column. **Figure 4** shows the extracted ion chromatogram (EIC) for the major glycan. The EIC shows that the glycan is split into a pair of peaks. This arises due to anomerization of carbon at the reducing end. The mass spectrum of the glycan is shown

in **Figure 5A** and the MS/MS spectrum of the glycan is shown in **Figure 5B**. Inspection of the spectrum reveals the diagnostic ion at m/z 204.085, which is the most intense ion in the MS/MS spectrum (Figure 5B). The possible fragment structures measured within a 2 ppm mass accuracy are also shown in Figure 5B (5).

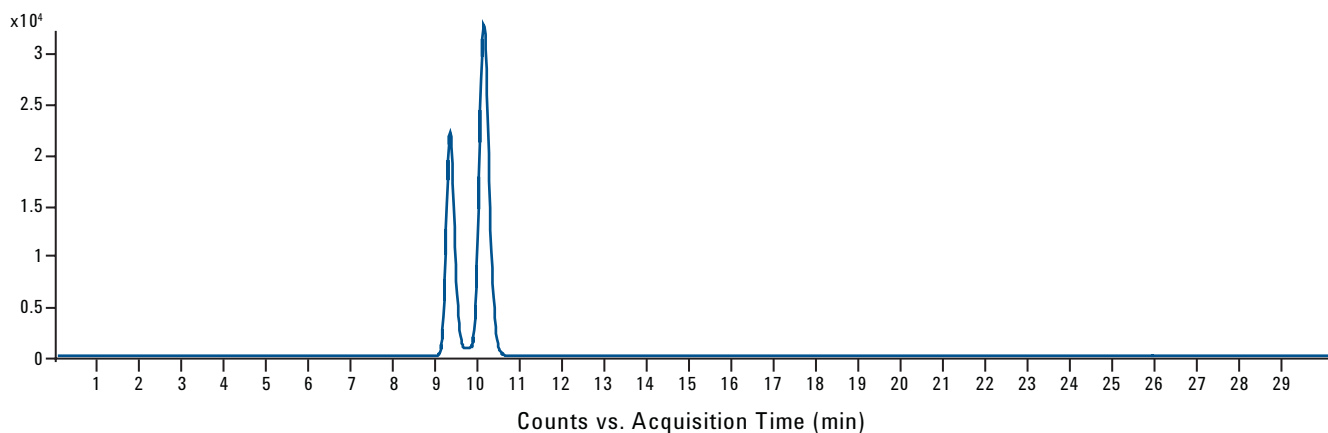


Figure 4: Extracted ion chromatogram (EIC) at m/z 732.28 ($[M+2H]^{+2}$) for the trypsin-digested mAb on a graphitized HPLC-Chip.

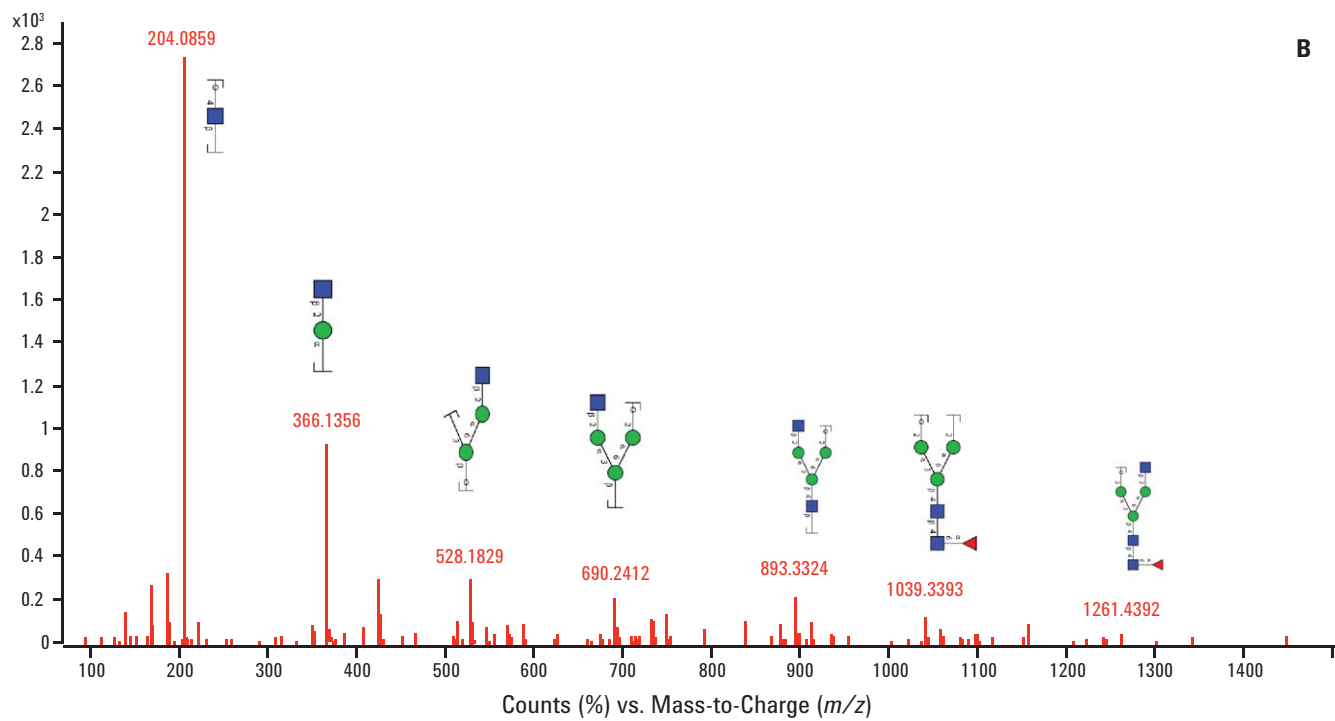
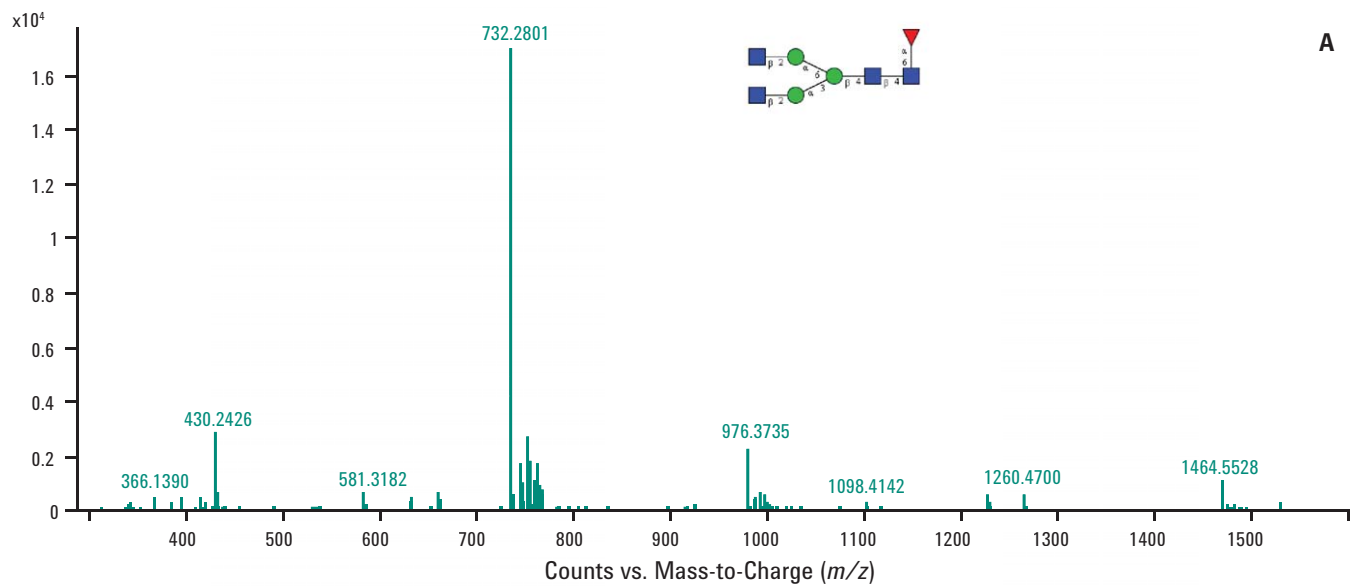


Figure 5: (A) Mass spectrum of the glycan with structure shown as inset. (B) MS/MS spectra of the glycan with the assigned fragment structures.

Conclusions

- The combination of the HPLC-Chip and the 6520 Accurate-Mass Q-TOF LC/MS with the BioConfirm software allows assignment of the glycopeptide from the mAb under study.
- MS/MS confirmation of a glycan moiety was made on the basis of the m/z 204 fragment.
- The glycan structure was determined by releasing the glycan from the peptide and eluting it on the Graphitized Carbon HPLC-Chip column.
- MS/MS of the glycan allowed interpretation to confirm the G0 biantennary structure.
- The flexible HPLC-Chip technology in combination with a Q-TOF is a valuable tool for studying glycoproteins.

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